A BIOCHEMICAL STUDY OF CELLULOSE SYNTHESIS BY *Acetobacter xylinum*

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INTRODUCTION

Cellulose, the structural material of plants, is formed from linear polymers of D-glucopyranose units. These units are connected by bonds between the one and four carbons of the glucose residue, the number one carbon having a β-configuration. This statement could be made with certainty only after years of research on cellulose structure.

The mechanism by which a living organism produces cellulose is unknown. In plants the study of this mechanism is complicated by the photosynthetic process. Certain bacteria, on the other hand, can convert hexoses into cellulose in a very short time and without the attendant problem of photosynthesis. They appear, therefore, to be efficient tools for the investigation of cellulose synthesis from the standpoint of convenience, simplicity and time.

The phenomenon of cellulose production by acetic acid bacteria was first reported by A. J. Brown in 1886.[4] Working with the "vinegar plant" which occurred in spoiled wine and beer, he isolated an organism to which he gave the name Bacterium xylinum, now known as Acetobacter xylinum. This organism produced a heavy membrane at the surface of suitable liquid media.

Brown treated the membrane to remove cellular and non-cellulosic materials and obtained a thin membrane which represented from 35 to 62 per cent of its original weight. Carbon and hydrogen analysis gave results in good agreement with values obtained with cellulose. Further

evidence that the material was cellulose was provided by its solubility in cupric ammonium hydroxide solution and its conversion to a reducing sugar when hydrolyzed with sulfuric acid.

Muenerling,[7] in 1899, questioned Brown's conclusion that the material was cellulose. He reported that the membrane was only slightly soluble in cupric ammonium hydroxide solution, that it contained from 2 to 3 per cent nitrogen and that crystals of glucosamine hydrochloride were obtained after hydrolysis of the membrane with concentrated hydrochloric acid. From these findings he concluded that the membrane was composed of chitin.

Subsequent workers, however, through physical and chemical examination have confirmed Brown's conclusion that the material was indeed cellulose. Some of these are summarized below.

C. A. Browne,[5] in 1906, examined cellulose which occurred in fermenting sugar cane juice. After boiling with 10 per cent caustic soda the membrane contained 0.2 per cent nitrogen and only 1 per cent was insoluble in cupric ammonium hydroxide solution. Hibbert and Barsha[1,12,13] subjected membranes from D-glucose, D-fructose and glycerol to various chemical transformations. Methylation, acetylation, acetolysis and hydrolysis yielded in each instance products identical with those obtained from cellulose of vegetable origin. Sutra[30] concluded from the nitrogen content of the specially treated membrane that it could not contain chitin.

Eggert and Luft,[6] working with dried membranes produced from sucrose, obtained X-ray diffraction diagrams which were similar to those

obtained with cotton linters. From analysis of X-ray diffraction data, Barsha and Hibbert[1] reported that membranes prepared from D-fructose, glycerol, sucrose, D-galactose and D-mannitol consisted of crystallites of cellulose. X-ray diffraction patterns identical with those of cotton linters were obtained by Khouvine, Chanpetier and Sutra[17] with membranes formed when glycerol, sorbitol and α-glucoheptitol were the substrates.

Further evidence for the identification of the membranes as cellulose has been provided by the infrared spectrophotometer[27] and the electron microscope.[8,9,15,23]

Acetobacter xylinum is capable of producing cellulose from a number of substrates. Table 1 lists the substrates which have been tested and the production or non-production of cellulose as reported by various workers. Contradictory reports have been made concerning some of the compounds. Thus Brown[4] obtained such a small yield from sucrose that he felt that it could be ascribed to impurities. Tarr and Hibbert[31] reported a yield of approximately 0.7 per cent from sucrose. However, the material was merely washed with hot water before drying and weighing, and no report was made of its positive identification as cellulose.

No synthesis of cellulose by Acetobacter xylinum from two-carbon compounds has been reported. None of the pentoses tested has been reported as yielding cellulose. Low yields from starch, levan, dextrin, lactose, maltose, mannose and rhamnose are subject to the same criticism as was applied in the case of sucrose. Substantial yields of cellulose were obtained from D-fructose, D-mannitol, glycerol, D-glucose and D-galactose, with yields decreasing in the order named.

TABLE 1

ORGANIC COMPOUNDS WHICH HAVE BEEN TESTED AS SUBSTRATES FOR CELLULOSE FORMATION IN CULTURES OF *A. xylinum*

Compound Tested	References[a] Reporting Cellulose	
	Present	Not Present
Starch	31	4
Inulin	31	
Levan	31	
Dextrin	31	
Raffinose		31
Cellobiose	27	11
Sucrose	31, 11	4
Lactose	31, 11	
Maltose	31, 11	
Glucose	31, 11, 27, 4	
Fructose	31, 11, 4	
Mannose	31	
Galactose	31, 11	
Sorbose		11
Xylose		31
Arabinose		31
Rhamnose	31	
α-Methyl glucoside		31
Ethanol		31, 10
Ethylene glycol		31
Glycerol	31, 11, 27, 17	
Erythritol	18	31, 11
Arabitol	18	
Mannitol	31, 4, 11, 18, 27	
Sorbitol	11, 18, 17	
Dulcitol	18	
Perseitol	18	
Volemitol	18	
α-Glucoheptitol	18, 17	
β-Glucoheptitol	18	
Tetraethylene glycol		31
Hexaethylene glycol		31
Acetic acid		11
Lactic acid	19	
Pyruvic acid		25
Glyceraldehyde	13	
α-Methyl glycerol		31
Glycerol chlorhydrin		31
l-Glucosan		31

[a] The numbers in this table correspond to numbered references in the bibliography.

Synthesis of cellulose by a resting cell suspension has been reported by Hestrin, Aschner and Mager.[11] A colloidal suspension of cells and cellulose fibrils was prepared from young Acetobacter xylinum membranes. The cells were separated from the fibrils by passing this suspension through cheese cloth. A cellulose membrane was produced when these cells were added to a thin layer of buffered substrate solution maintained at 37°C. Cellulose production without increase in cell number had apparently been obtained since Acetobacter xylinum does not multiply at this temperature even in nutrient solution.

The formation of cellulose from substrates labeled with radioactive carbon-14 has been investigated in recent research. The results of an experiment using D-glucose-1-C^{14} has been published by Minor, Greathouse, Shirk, Schwartz and Harris.[22] Hydrolysis of the cellulose and degradation of the resulting glucose revealed that the major portion of the activity of the glucose residues was in the one position (Table 2).

TABLE 2

LOCATION OF LABEL IN STRUCTURE OF GLUCOSE UNITS IN BACTERIAL CELLULOSE

Position	% of Label
1	82.2
2	0
3	10.8
4	8.7
5	0
6	0

The specific activity of the cellulose was 0.071 microcuries per milligram as compared to 0.34 microcuries per milligram in the glucose-1-C^{14} which had been supplied (Table 3).

TABLE 3

SPECIFIC RADIOACTIVITY VALUES OF CULTURE PRODUCTS

	Microcuries/mg. C
D-Glucose-1-C^{14} supplied	0.34
D-Glucose-C^{14} obtained	0.071
CO_2	0.37

The authors calculated from the ratio of the specific radioactivities of cellulose to glucose (21 per cent) and the amount of C^{14} located at position one of the glucose (82.2 per cent), that at most 17 per cent of the cellulose could have been formed from the initial D-glucose-1-C^{14} molecule by direct polymerization.

Synthesis of cellulose by microörganisms seems to be confined almost exclusively to the genus Acetobacter. Beijerinck,[2] in 1898, found that Acetobacter pasteurianum and Acetobacter xylinum produced slimes which gave a positive cellulose reaction. Van Wisselingh[32,24] tested one hundred different fungi, including bacteria, yeasts and molds, and concluded that cellulose was not commonly found in bacteria other than Acetobacter xylinum. Kaushal and Walker,[14] in 1951, reported positive cellulose reactions in pellicles formed by Acetobacter acetigenum, Acetobacter pasteurianum and Acetobacter kutzingianum. The cellulose test normally employed depended on the formation of a blue color when the material was treated with iodine-zinc chloride solution or with 75 per cent sulfuric acid and iodine-potassium iodide solution.

The members of the genus Acetobacter are described in Bergey's Manual[3] as having ellipsoidal to long and rod shaped cells occurring singly, in pairs, or in short or long chains. Young cells are Gram-negative; old cells often are Gram-variable. The organisms are obligate aerobes, usually strongly catalase-positive, and oxidize various organic compounds to organic acids and other products which may undergo further oxidation. The cells of Acetobacter xylinum are described as rods, about two microns long, which occur singly and in chains. They have a shiny envelope which gives the cellulose reaction. Acids are produced from glucose, ethanol, propyl alcohol and ethylene glycol. No acid is produced from arabinose, fructose, galactose, maltose, lactose, raffinose, dextrin, starch, methyl alcohol, isopropyl alcohol, butyl alcohol, isobutyl alcohol, amyl alcohol, mannitol or acetaldehyde.

A medium consisting of yeast water or yeast extract, a carbohydrate substrate and phosphate, adjusted to a pH between five and six, has been widely used for cellulose production. The cultures were incubated at temperatures of 28-32°C. for periods ranging up to twenty-one days. Tarr and Hibbert[31] obtained cellulose from media in which ammonium salts, urea or L-asparagine had been substituted for yeast extract. The addition of ethanol to the media was found to increase the cellulose yield although cellulose was not produced when ethanol was the only carbon source.

Many problems concerning the metabolism of Acetobacter xylinum and its synthesis of cellulose remain unsolved. Even the fundamental

question of what substrates can be converted to cellulose is confused by conflicting reports in the literature. The effects of variation of the concentrations of media components, of pH and of oxygen supply are unknown. The requirements of the organism for growth factors, the nitrogen sources utilized, the pathways for carbohydrate metabolism have been partially established. The problems which are, perhaps, the most interesting and difficult have barely been touched upon. These are the isolation of the enzyme system responsible for cellulose synthesis and the elucidation of the mechanism of cellulose formation.

The application of radioactive substrates to studies of cellulose synthesis by Acetobacter xylinum has made economy an important reason for determining the conditions necessary for maximum cellulose production. The research reported in this dissertation was undertaken to improve the yield of cellulose from glucose and to make beginnings toward the solution of some of the problems set forth above.

CHAPTER I

A REVIEW OF EXPERIMENTAL METHODS AND MATERIALS

The strain of A. xylinum used in this research for the production of cellulose was supplied by Dr. G. A. Greathouse and Dr. H. G. Shirk of the Prevention of Deterioration Center, National Research Council, Washington, D. C. The organisms were propagated in a liquid medium, hereafter referred to as the "standard medium," which had the following composition:

Yeast Extract	1 per cent
Glucose	1 per cent
KH_2PO_4	0.3 per cent (0.022 M)
pH	6.0 ± 0.1

Efforts were made to make transfers to fresh media at 2 or 3 day intervals. Occasionally, the appearance of contamination made it necessary to revert for an inoculum to an older, uncontaminated culture. Therefore, the history of the inocula, from the standpoint of regularity of transfers, varied throughout these experiments.

The stock cultures were incubated at 30°C., as were all of the experimental cultures. A Thelco cabinet type incubator was used. The incubator was located in an air conditioned room maintained at a temperature of approximately 28°C.

In early experiments media were sterilized by filtration of the complete medium through a Seitz filter. Media containing yeast extract filtered slowly, and this was believed to be responsible for the presence

of contamination in instances where a nonsterile medium was obtained. To avoid this difficulty a solution of yeast extract and phosphate was sterilized in an autoclave for 20 min. at 15 lbs. pressure. The glucose solution, which passed rapidly through the filter, was sterilized separately by filtration. The two solutions were subsequently combined to make the complete medium.

One final modification was made in the sterilization procedure. The glucose, as a 40 per cent solution, was also sterilized in the autoclave for 20 min. at 15 lbs. pressure. This method was more convenient and faster than filtration. There was never an indication that the method of sterilization had an effect on cellulose production.

All chemicals employed in the preparation of media or for other purposes were used as received from the manufacturer and without further purification. A list of these chemicals and their sources appears in Appendix II.

Test tubes were used for growing cultures in preliminary experiments in which qualitative observations were made. Erlenmeyer flasks, 250 ml. capacity, served as culture vessels in experiments where quantitative measurements were made. Twenty milliliters of medium in these flasks provided a culture surface for membrane formation which was approximately 7.5 cm. in diameter. This volume of medium was used consistently for making quantitative determinations since it yielded a membrane which could be conveniently handled.

The methods of inoculation used in this research varied with the type of medium employed. Changes in these methods were also made

to obtain uniformity in the inocula. The procedures are outlined below:

(1) A portion, usually 1 ml., from a stock culture was transferred to the test medium. The ages of these inocula varied.

(2) A suspension of washed cells, prepared from a stock culture, was used as inoculum.

(3) A suspension of cells, prepared from the growth on an agar slant, served as the inoculum. The slants contained yeast extract (2 per cent), carbohydrate substrate (2 per cent), KH_2PO_4 (0.3 per cent) and agar (2 per cent). They were inoculated by passing the membrane from a recently inoculated (1 to 3 days) stock culture over the surface of the slant. Sterile distilled water was added to the slants after 3 days incubation and the cells loosened and suspended in the water by means of an inoculating loop.

(4) The organisms were grown on slants as in (3) and a suspension of washed cells was prepared for the inoculum.

The term "washed cells" as used in this dissertation refers to cells which have been alternately suspended in sterile, distilled water and "spun down" in the centrifuge. This process, repeated three or four times with fresh volumes of water, constitutes the "washing." The final suspension served to inoculate synthetic media.

The inoculating technique was not standardized in early experiments. Stock cultures of various ages were used directly as inocula. The question of what effect the age of the inoculum had upon cellulose production was investigated in the following manner. Six flasks, each containing 20 ml. of the standard medium, were inoculated by 1 ml. transfers from a single, 1 day old, stock culture. After 4 days incubation, and subsequently at 4-day intervals for a total period of 24 days, flasks containing this same medium were inoculated with 1 ml. transfers from one of the six original cultures. Cellulose yields were determined in the cultures four days after inoculation.

Media which had received inocula 4, 8 and 12 days old, produced approximately the same amount of cellulose. A definitely lower yield was obtained from inocula which were more than 12 days old (Table 4).

The need for a standardized technique of inoculation led to an examination of the effect of the size of the inoculum on cellulose production. A heavy suspension in sterile distilled water was prepared from a 5-day growth on agar slants. Various dilutions were made of the original suspension, and duplicate flasks of the standard medium were inoculated with 1 ml. portions of each dilution. The dilutions were expressed (Table 5) as the ratio of the final volume of the dilution to the volume of the original suspension used in the dilution. These values ranged from 1 to 400. Membranes were removed from the cultures five days after inoculation and the weight of the cellulose was determined.

TABLE 4

THE INFLUENCE OF THE AGE OF THE INOCULA ON CELLULOSIC PRODUCTION BY *A. xylinum*

Age of Inocula (days)	pH (final)	Cellulose Produced (mg.)	Age of Inocula (days)	pH (final)	Cellulose Produced (mg.)
4	4.8	14.8	16	4.8	11.9
	4.7	16.7		4.8	11.3
	4.6	17.2		4.8	12.4
8	4.9	16.9	20	4.9	11.2
	4.8	15.9		4.7	11.4
	4.7	16.6		4.8	11.4
12	4.7	15.1	24	5.1	8.4
	4.8	14.4		5.8	9.2
	4.8	15.7		5.5	10.7

Medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 6

Inocula: One milliliter each from cultures in the above medium which had been incubated the number of days indicated in the table

Incubation: Four days at 30°C.

TABLE 5

THE INFLUENCE OF THE SIZE OF INOCULA ON CELLULOSE PRODUCTION BY *A. xylinum*

Dilution Factor[a]	Cellulose Produced (mg.)	Dilution Factor[a]	Cellulose Produced (mg.)
1	12.3 10.4	40	11.9 9.0
4	10.3 11.8	100	9.8 9.9
8	12.7 10.7	200	12.9 11.5
16	11.6 12.4	400	14.5 10.8
30	11.1 13.7		

[a]Dilution factor: $\frac{\text{Vol. of dilution}}{\text{Vol. of suspension diluted}}$

Medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 6

Inocula: One milliliter portions of the dilutions of a suspension of the five-day growth on agar slants

Incubation: Five days at 30°C.

There was no difference in the amount of cellulose produced in the cultures that could be ascribed to differences in the size of the inocula. Although the yield of cellulose after 5 days incubation was fairly uniform in all the cultures, membranes were observed to form much more rapidly in the cultures which had received heavy inocula. This indicated that inoculum size had a definite influence on the rate of cellulose production.

One milliliter portions of suspensions of cells taken from agar slants were used as inocula in the majority of the experiments. A twenty-five fold dilution of these inocula gave approximately 90 per cent transmission in the Evelyn Photoelectric Colorimeter using a 540 millimicron filter. A suspension of cells with a density equal to that of the inocula contained about 0.14 mg./ml. of nitrogen. Cultures in the standard medium reached maximum cellulose production within 48 hours after receiving the inocula of this size.

The membranes produced by A. xylinum forms at the surface of the culture. Thin and transparent in the early stages of growth, it becomes white and opaque as it gradually thickens with age. When removed from the culture it is heavy and swollen with absorbed water. The undersurface is coated with a clear, jelly-like material. If the membrane is washed well with water and dried by treatment with acetone, the resulting film is soft and semi-transparent, greatly resembling lens tissue in texture and appearance.

The membranes contained a large proportion of nitrogenous material, and their treatment before weighing was designed to remove

noncellulosic substances. As they were removed from the cultures the membranes were pressed against the neck of the flask to remove a portion of the absorbed medium. An initial washing operation was performed by a prolonged (12 to 24 hours) passage of tap water over the membranes which were retained in a Buchner funnel.

The next operation involved heating the membranes in a 1 per cent NaOH solution. This was accomplished at first by boiling the membranes in the NaOH solution under reflux. A more convenient method, which was used almost exclusively, consisted of heating the membranes in 1 per cent NaOH for two hours at 15 lbs. pressure. The digestion in NaOH was followed by a final wash in tap water which was continued until the membranes no longer gave an alkaline reaction with litmus paper. This required approximately four hours. The membranes were finally suspended from nichrome wires, dried and weighed. The drying process required 12 hours or more at room temperature or 3 to 4 hours in an oven at 75°C.

The calculation of cellulose yields was based on the weight of cellulose which would be formed by the complete conversion to cellulose of the substrate in the medium. The anhydroglucose unit of the cellulose chain has a molecular weight of 162. Thus, 180 mg. of glucose or fructose or 182 mg. of mannitol could theoretically form 162 mg. of cellulose. The following formula was used for making the calculation of the cellulose yield from glucose:

$$\frac{\text{mg. cellulose formed}}{\text{mg. glucose in medium} \times \frac{162}{180}} \times 100 = \text{per cent of theoretical yield of cellulose}$$

An estimate of the efficiency of the digestion was obtained in the following experiments. Three membranes, formed under the same cultural conditions, were washed as usual in water. Their dry weight was determined and then one membrane was subjected to the complete treatment described above, while nitrogen determinations (Micro-Kjeldahl method) were performed on the other two membranes. The average nitrogen content of six untreated membranes which were produced in media at three different initial pH values was 2.5 mg., or roughly 8 per cent (Table 6). Approximately 40 per cent of the original membrane weight remained after NaOH digestion.

Nitrogen determinations were also performed on completely treated membranes which were formed in media of various initial pH values. Four membranes produced in cultures of the same initial pH value were combined for nitrogen analysis. The nitrogen content ranged from 0.05 mg. in the smallest membranes to 0.38 mg. in the four largest membranes (Table 7). This indicated that approximately 96 per cent of the nitrogen in the membranes was removed by the digestion described above.

TABLE 6

THE NITROGEN CONTENT OF UNTREATED MEMBRANES FROM CULTURES OF *A. xylinum*

pH[a] (initial)	Membrane Weight (mg.)	Nitrogen[b] (mg.)	Nitrogen[b] (%)	Cellulose Produced (mg.)
6.5	33.1	2.7	8.2	....
	30.1	2.5	8.0	....
	33.0	...	...	12.8
7.0	34.6	3.0	8.6	....
	30.7	2.3	7.4	....
	34.1	...	...	13.3
7.5	28.9	2.2	7.6	....
	28.2	2.1	7.3	....
	28.7	...	...	11.7

[a]The pH of the media was adjusted with KOH. [b]Nitrogen determinations were made by the Micro-Kheldahl method.

Basal medium: Yeast extract . . . . . 1 per cent

Glucose . . . . . 1 per cent

Phosphate . . . . . 0.022 molar

Inocula: One milliliter portions from a suspension of a 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

TABLE 7

THE NITROGEN CONTENT OF MEMBRANES FROM CULTURES OF *A. xylinum* AFTER DIGESTION WITH SODIUM HYDROXIDE[a]

pH[b] (initial)	Cellulose Produced[c] (mg.)	Nitrogen[d] (mg.)	Nitrogen[d] (%)
4	18.3	0.05	0.3
5	35.4	0.24	0.7
6	45.4	0.36	0.8
7	68.3	0.38	0.6
8	44.8	0.23	0.5

[a]Other data from this experiment appear in Table 8. [b]The pH of the media was adjusted with KOH. [c]The weights represent the combined cellulose from four cultures. [d]Nitrogen determinations were made by the Micro-Kjeldahl method.

Basal medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent
Phosphate . . . . . 0.022 molar

Inocula: One milliliter portions from a suspension of 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

CHAPTER II

FACTORS INFLUENCING THE PRODUCTION OF CELLULOSE BY *Acetobacter xylinum* IN MEDIA CONTAINING YEAST EXTRACT

The Influence of the pH of the Medium on Cellulose Production

It was observed, while working with media containing glucose, that the pH of the cultures decreased rapidly after inoculation. A minimum was reached when the cultures were approximately 24 hours old. This was followed by a slow increase until the values remained essentially constant. The deceleration of the rate of the decrease in the pH values and the subsequent increase occurred during the period of maximum cellulose production in the culture. This behavior suggested that a relationship existed between cellulose production and the pH of the culture. The observed minimum in the pH values might, therefore, represent the hydrogen ion concentration best suited to cellulose production.

The relation between the initial pH of the culture and the amount of cellulose produced was investigated in the following manner. Yeast extract (1 g.) was dissolved in water (75 ml.) containing H_3PO_4 (0.63 ml.; 0.325 M). This solution was adjusted to the desired pH value with KOH and sufficient distilled water was added to make a final volume of 95 ml. A 19 ml. portion of this solution was placed in each of four flasks and sterilized in the autoclave. Losses in weight through

evaporation were remedied by the addition of sterile water. One milliliter of a 20 per cent glucose solution (sterilized in the autoclave at 40 per cent concentration) was added to each flask. Media having initial pH values of 4, 5, 6, 7, 8 and 8.5 were prepared in this manner. A three-day growth on agar slants was used to make a cell suspension from which 1 ml. volumes were taken as inocula.

Membranes were removed from the cultures after incubating for 48 hours. These were dried and weighed after treatment in an autoclave with 1 per cent NaOH as described in Chapter I. The residual media in each group of four cultures of the same initial pH value were combined and diluted to 80 ml. Final pH values were obtained from these composites.

The amount of cellulose produced increased as the initial pH readings of the media were increased from 4 to 7 but decreased in cultures which had an initial pH of 8 (Table 8). No growth or cellulose production occurred in cultures at pH 8.5. Final pH values followed the same general pattern as the cellulose yields, reaching a maximum in cultures of initial pH 7.

The increase in cellulose production noted above might conceivably have been due to factors other than the concentration of hydrogen ion. The concentration of the potassium ion was necessarily increased as the initial pH was adjusted to higher values. This increase in potassium ion concentration may have had some stimulatory effect on cellulose production. Also, since the incubation period had not been extended beyond 48 hours, the possibility remained that maximum

TABLE 8

THE INFLUENCE OF THE pH OF THE MEDIUM ON CELLULOSE PRODUCTION BY *A. xylinum*

pH[a] (initial)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
4	3.3	4.6 4.2 4.7 4.8	2.6
5	3.75	9.0 9.1 9.0 8.3	4.9
6	4.0	11.3 11.1 11.5 11.5	6.3
7	5.1	17.3 17.2 16.5 17.3	9.5
8	4.4	11.1 10.0 11.7 12.0	6.2
8.5	7.1	0	

[a]The pH of the media was adjusted with KOH.

Basal medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent
Phosphate . . . . . 0.022 molar

Inoculum: One milliliter transfers from a suspension of a 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

production of cellulose had not been reached in all of the cultures.

These doubts were substantially dispelled by an experiment which involved both of the above factors. A medium which contained only glucose (1 per cent) and yeast extract (1 per cent) was prepared. The pH (6.8) of part of this medium was not altered. Other portions were adjusted to pH 3.5 and pH 4.65 by the addition of H_3PO_4 to one and H_3PO_4 and KOH to the other. Twenty milliliter volumes at each pH value were inoculated with cell suspension.

Membranes were removed after 12, 24, 36, 48 and 96 hours incubation. These were processed and weighed as in the preceding experiment. The final pH value (pH of the medium at the time the membrane was removed) was determined on individual cultures without diluting to the original volume.

The yield of cellulose from cultures inoculated at pH 6.8 was markedly higher than from the cultures having lower initial pH values (Table 9). Thus, an increased amount of cellulose was produced at the high pH although no potassium ion or phosphate had been added. Also, the weight of cellulose present after 96 hours incubation did not differ significantly from the amount found after 48 hours incubation. Maximum production of cellulose had been obtained within 48 hours even in cultures with a low initial pH. This has been the case consistently in glucose media with the rather large inocula used in these experiments. Since the inoculum in the previous experiment was of a similar size, it is believed that there, also, maximum yields were obtained.

TABLE 9

THE INFLUENCE OF THE pH OF THE MEDIUM ON THE RATE OF CELLULOSE PRODUCTION BY *A. xylinum*

Incubation Period (hours)	pH (final)			Cellulose Produced (mg.)			Cellulose Yield (Avg.%)		
	a	b	c	a	b	c	a	b	c
24	3.3	3.5	3.9	3.4	8.6	8.9	1.7	4.7	5.0
	3.3	3.6	3.9	2.8	7.9	8.8			
	3.3	3.6	3.9	3.2	9.1	9.4			
36	3.2	3.7	4.1	4.5	11.5	13.7	2.6	6.3	7.4
	3.2	3.7	3.9	4.6	11.2	12.2			
	3.2	3.7	4.15	4.6	11.5	14.2			
48	3.3	3.6	4.6	6.0	12.4	17.4	3.1	6.8	9.4
	3.3	3.6	4.5	5.3	12.6	16.0			
	3.2	3.7	4.6	5.3	11.9	17.3			
96	3.3	3.9	4.85	5.4	10.1	14.6	3.2	5.6	8.5
	3.3	3.9	4.85	5.9	10.2	16.1			
	3.3	3.9	4.85	6.0	10.0	15.1			

[a]Basal medium made 0.022 N in H_3PO_4, pH 3.5. [b]Basal medium made 0.009 N in H_3PO_4 and 0.013 N in KH_2PO_4, pH 4.65. [c]Basal medium made with no phosphate added, pH 6.8.

Basal medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants

Incubation: At 30°C.

No growth was obtained when the stock culture medium (p. 9) was adjusted to pH 7 and inoculated by a 1 ml. transfer from another stock culture. Powell also failed to obtain growth in media at initial pH 7.[25] It is possible to explain this apparent contradiction to the results obtained with cultures at an initial pH of 8 (Table 8) on the basis of the size of the inocula. There might be enough active, acid-producing enzyme present in a large inoculum to reduce the high pH value quickly to one at which the organism can survive and grow; but with comparatively small inocula, as in the case of the stock culture, this might not occur. In the cultures with an initial pH of 8.5 (Table 8) this reduction in pH may not have proceeded to a point where the organisms would live.

The increased cellulose yields obtained in cultures with high initial pH values might be due to an increase in the reaction rate of the cellulose producing enzymes. The effect might also be caused by an increase in the amount of the cellulose producing enzyme, with or without a concurrent increase in cell number. In the latter case, the sudden change of an actively growing culture from a low to a high pH should not be expected to result in a marked or immediate increase in cellulose production.

A medium containing 1 per cent glucose and 1 per cent yeast extract was adjusted to pH 4.4 with H_3PO_4 and KOH. The customary 20 ml. volumes of the medium were inoculated from a cell suspension and incubated for 24 hours. At this time, membranes were removed from three cultures and the residual media adjusted to approximately

pH 7. A volume of 3.25 ml. of KOH solution was required by each culture to raise the pH from 3.5 to 7.0 ± 0.1. This same volume of KOH solution was added to each of nine of the eighteen cultures remaining in the incubator. Each of the other nine received an equivalent volume of sterile, distilled water.

Cultures of each type were removed after 48, 96 and 136 hours of incubation. Final pH values were determined and membranes processed at the end of each incubation period.

The weight of cellulose in the pH-adjusted cultures after 48 hours of incubation was more than double that obtained in unadjusted cultures of the same age (Table 10). An additional average increase of 47 per cent (6.3 mg.) was shown by the pH-adjusted cultures at 96 hours, whereas the cellulose in the unadjusted cultures had increased 25 per cent (1.2 mg.) in the same period. This was the maximum production since the cultures removed at 136 hours showed no increase in cellulose. [The increase in cellulose in the pH 4.4 cultures beyond 48 hours, where maximum yield was normally obtained, may have been due to the availability of fresh culture surface after the submergence of the membranes when water was added.]

The pH in the adjusted cultures, after decreasing from 7 to 5.5 in 24 hours, increased during the remaining incubation period, reaching pH 8.5 in the 136-hour cultures. This was the highest value of the final pH noted in the course of this research.

The previously mentioned failure to obtain growth in cultures at pH 7 [This observation needs further confirmation.] may indicate

TABLE 10

THE EFFECT ON CELLULOSE PRODUCTION OF A POST-INOCULATION ADJUSTMENT OF THE pH OF CULTURES OF *A. xylinum*

Incubation Period (hours)	Unadjusted Cultures			Adjusted Cultures[a]		
	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
24	3.5	3.2	1.8	...	....	....
	3.6	3.2		...	....	....
	3.5	3.5		...	....	....
48	3.35	4.8	2.7	5.5	12.9	7.4
	3.35	4.9		5.6	13.7	
	3.35	5.0		5.4	13.2	
96	3.4	5.9	3.4	7.5	19.2	10.9
	3.65	5.7		8.5	19.5	
	3.6	6.7		7.5	20.1	
136	3.7	5.2	2.9	8.5	18.5	10.6
	3.6	4.7		8.5	19.1	
	3.6	5.7		8.7	20.4	

[a]The pH of these cultures was adjusted to pH 7 with KOH after 24 hours of incubation.

Medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent
Phosphate . . . . . 0.022 molar
pH 4.4

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants

Incubation: At 30°C.

that the optimum pH for the growth of A. xylinum is below this value. It does not seem logical on this basis that adjustment of the pH of the growing culture from 3.5 to 7 would result in increased cell number. If this reasoning is correct the increased production of cellulose experienced above should reflect a higher cellulose yield per cell rather than one due to a larger population. This assumption could not be confirmed without a method of relating growth and cellulose production.

The Influence of the Concentration of the Components of the Media on Cellulose Production

New incentive for establishing optimum conditions for cellulose production by A. xylinum was provided by the use of glucose-1-C^{14} in studies of cellulose formation by this organism.[22] Considering the cost of specifically labeled glucose, conditions which gave a maximum yield of cellulose from a minimum amount of the hexose would obviously be the most economical. These conditions would include, among others, optimum concentrations of the components in the medium.

Cellulose yields from cultures in the standard medium (p. 9) had varied in these experiments from 6 to 10 per cent. The influence of the concentration of each component upon the amount of cellulose formed was determined by varying the concentration of one component while that of the others was held constant. The effect of glucose concentration was first to be investigated in this manner.

A solution of yeast extract and KH_2PO_4 was sterilized in the autoclave. Sterile water and glucose solution, in quantities which gave glucose concentrations ranging from 0 to 10 per cent were added to two flasks

of this medium. The concentrations of yeast extract (1 per cent) and KH_2PO_4 (0.3 per cent) were the same as in the standard medium, as was also the pH (6). The media were inoculated with cell suspension and incubated for 48 hours. The membrane weight and final pH value were determined in each culture.

The actual weight of cellulose was lowest (1.3 mg.) in cultures with no glucose added (Table 11). The membrane weight then increased, reached a maximum at 0.5 to 1 per cent glucose concentration and decreased as the glucose content of the media increased.

The exact source of the cellulose that formed in cultures to which no glucose had been added was not known. It may have been due to the presence in the yeast extract of a slight amount of hexose. Although the same quantity of yeast extract was present in each culture, there appeared to be little reason in calculating cellulose yields to take into consideration the cellulose production which might be attributed to it. The membrane weight reported for the other cultures was not, therefore, reduced by this amount.

Cultures which contained 0.2 per cent glucose gave the highest value (31 per cent) of the cellulose yield (Table 11). The yields from culures of increasing glucose content decreased rapidly from this value to the lowest value (0.4 per cent) which occurred in the culture containing 10 per cent glucose. The weights of the cellulose membranes produced in cultures with 3, 6 and 10 per cent glucose were approximately the same.

TABLE 11

THE INFLUENCE OF THE CONCENTRATION OF GLUCOSE IN THE MEDIUM ON CELLULOSE PRODUCTION BY *A. xylinum*

Glucose Concentration (%)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
0	6.5	1.3	...
	6.5	1.4	...
	6.5	1.3	...
0.2	6.4	9.9	31
	6.45	10.0	
	6.45	13.7	
0.5	6.1	12.3	13.8
	6.0	12.7	
	6.1	12.1	
1.0	4.3	12.4	6.6
	4.1	11.2	
	4.25	12.1	
3.0	3.2	6.7	1.2
	3.2	6.6	
	3.2	6.6	
6.0	3.0	7.8	0.7
	3.0	7.0	
	3.0	7.2	
10.0	3.0	7.6	0.4

Basal medium: Yeast extract . . . . . 1 per cent

KH_2PO_4 . . . . . 0.3 per cent

pH 6

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

Final pH values decreased in the same order as the cellulose yields. They dropped from a high of 6.5 in the cultures which contained no added glucose to a low of 3.0 in the cultures which contained 6 and 10 per cent glucose. The values at 0 and 0.2 per cent glucose presented a rise over the pH of the original media. There was evidently an increased production of acid in cultures of high glucose content. The correspondence between the final pH values and the cellulose yields indicates again the relation between these phenomena.

Yeast extract or yeast water has a well known ability to stimulate the growth of many microörganisms. Yeast water was used in media for growing A. xylinum in the original experiments of Brown.[4] Different preparations of yeast water, and probably of yeast extract, differ in their effect on cellulose production. Thus Brown obtained only 3.8 mg. of cellulose from 100 ml. of his yeast water preparation with no carbon source added, while Tarr and Hibbert[31] reported an average yield of 66 mg. from 50 ml. of their yeast water preparation. This variability of the material limits the value of a determination of the concentration optimum for yeast extract. However, yeast extract from a single commercial source was used in the experiments reported in this dissertation.

Yeast extract has a buffering action, and it was necessary to vary slightly the ratio of KH_2PO_4 to K_2HPO_4 as the concentrations of yeast extract were increased in media prepared for this experiment. The media were adjusted to pH 6.2 $\pm$ 0.1. Glucose concentration was held constant at 1 per cent, and phosphate was 0.022 M in all media. The yeast extract concentrations varied from 0.01 to 5 per cent.

No cellulose was produced in media containing 0.01 per cent yeast extract (Table 12). The yields increased from 0.3 per cent (0.1 per cent yeast extract) to a maximum of 9.5 per cent (2.5 per cent yeast extract) and decreased to 6.9 per cent (5 per cent yeast extract).

Again, the final pH values followed a trend similar to that of the cellulose yields. The amount of cellulose formed was probably partially controlled by the increased buffering action of the yeast extract with increasing concentration.

A previous experiment (Table 9) had demonstrated that phosphate concentration had no profound effect on cellulose yield. This was verified in an experiment in which glucose and yeast extract concentrations were held constant. The concentration of phosphate was varied by adding increasing quantities of H_3PO_4 to portions of the medium and adjusting to pH 6.6 $\pm$ 0.1 with KOH solution. This value of pH was chosen since the medium without added phosphate was found to be at pH 6.7, and previous experience had shown that better yields were obtained with an initial pH higher than the pH 6 normally employed.

After inoculation and incubation for 48 hours the cellulose yield was 6 per cent in cultures without added phosphate (Table 13). A maximum of 8 per cent cellulose was obtained in cultures containing 0.088 M phosphate, the highest concentration represented. The same correlation between cellulose yields and final pH values that had been noted in the experiments with glucose and yeast extract appeared here. The buffering action of the phosphate evidently influenced cellulose production.

TABLE 12

THE INFLUENCE OF THE CONCENTRATION OF YEAST EXTRACT IN THE MEDIUM ON THE PRODUCTION OF CELLULOSE BY *A. xylinum*

Yeast Extract Concentration (%)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
0.01	3.7	0	...
	3.6	0	...
	3.6	0	...
0.1	3.5	0.4	0.3
	3.5	0.6	
	3.5		
0.5	3.75	10.4	5.2
		9.1	
		8.5	
1.0	4.5	14.6	8.1
	4.6	13.8	
	4.6	15.1	
2.5	5.5	16.8	9.5
	5.3	16.9	
	5.3	17.4	
5.0	5.6	12.1	6.9
	5.5	12.3	
	5.3	12.7	

Basal medium: Glucose . . . . . 1 per cent

Phosphate . . . . . 0.022 molar

pH . . . . . 6.2 ± 0.1

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

TABLE 13

THE INFLUENCE OF THE CONCENTRATION OF PHOSPHATE IN THE MEDIUM ON THE PRODUCTION OF CELLULOSE BY *A. xylinum*

Phosphate Concentration (molar)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (avg.%)
0	3.8	11.1	5.9
	3.9	8.9	
	3.9	11.8	
0.0011	3.8	11.7	7.2
	3.9	12.2	
	3.9	12.0	
0.011	3.9	13.4	7.4
	4.0	13.7	
	4.0	12.9	
0.022	4.3	13.7	7.1
	4.5	13.0	
	4.2	11.7	
0.044	4.4	15.3	8.2
	4.4	14.7	
	4.4	14.5	
0.088	4.7	14.2	8.1
	4.7	14.4	
	4.7	15.2	

Basal medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent
pH . . . . . 6.6 ± 0.1

Inocula: One milliliter transfers from a suspension of 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

Ethanol, when added to media containing glucose, increased cellulose formation. No cellulose, however, was obtained from synthetic media containing asparagine and ethanol alone as sources of carbon.[31] Membranes produced in cultures containing glucose and ethanol-1-C^{14} or ethanol-2-C^{14} were not radioactive.[10] Thus, the inclusion of ethanol in media containing glucose seems to make more glucose available for the synthesis of cellulose.

Conditions considered to be most favorable for cellulose production were employed in investigating the influence of ethanol concentration on cellulose yield. The basal medium contained 0.2 per cent glucose and 2.5 per cent yeast extract. An initial pH of 6.7 was obtained with 0.044 molar phosphate. Ethanol concentrations in four sets of media were 0, 0.6, 1.2 and 2.4 per cent.

The cellulose yield after 48 hours incubation was 21 per cent in cultures without ethanol (Table 14). This increased to 43.6, then dropped to 40.6 per cent in cultures which contained, respectively, 1.2 and 2.4 per cent ethanol. These values were calculated entirely on the basis of the glucose present.

The correlation between cellulose yields and final pH values observed in the three preceding experiments was not reproduced in the case of ethanol. Although the presence of ethanol in the cultures resulted in lower values of the final pH, the stimulation of cellulose formation by the ethanol apparently outweighed the adverse effects of pH.

TABLE 14

THE INFLUENCE OF THE CONCENTRATION OF ETHANOL IN THE MEDIUM ON THE PRODUCTION OF CELLULOSE BY *A. xylinum*

Ethanol Concentration (%)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg.%)
0	6.9	8.1 7.1 8.1	21
0.6	6.4	13.5 14.5 13.4	38.4
1.2	4.6	15.2 16.4 15.6	43.6
2.4	4.3	14.7 13.9 13.1	40.6

Basal medium: Yeast extract . . . . . 2.5 per cent
Glucose . . . . . 0.2 per cent
Phosphate . . . . . .0.022 M
pH 6

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

The Rate of Cellulose Formation in Cultures of *Acetobacter xylinum*

Long periods of incubation have generally been employed for the production of cellulose by *A. xylinum*. Brown[4] incubated his cultures for 16 days. Khouvine[16] used incubation periods up to 21 days. Tarr and Hibbert[31] reported that 10 days were necessary for maximum cellulose production.

Certain disadvantages are associated with the use of lengthy incubation times. The delay in obtaining data prolongs the research. Requirements for space and equipment are increased. Furthermore, moisture loss from the cultures during incubation is considerable and causes variation in the concentrations of media components.

The primary factor controlling the rate of cellulose production is probably the size of the inoculum. This was indicated by observations made in connection with the experiment relating cellulose yield to the size of the inoculum (Table 5). The desirability of reducing the incubation period led to a study of the rate of cellulose formation when a large inoculum was employed.

Glucose and mannitol were used as carbohydrate substrates in separate media for studying the rate of cellulose formation. The media each contained yeast extract (1 per cent), carbohydrate (1 per cent) and KH_2PO_4 (0.3 per cent) at a pH of 6.0 ± 0.1. The organisms for the inocula (cell suspensions) had been propagated in broth culture and on slants which contained the same carbohydrate as the medium they served to inoculate. Cellulose yields and final pH values were determined at intervals during the incubation period.

A small amount of cellulose formed in both types of media during the first 12 hours of incubation (Tables 15 and 16). Cellulose formation proceeded at a more rapid rate in the medium containing glucose. The maximum yield of cellulose in the glucose medium was obtained in 48 hours or less. Maximum yield in the mannitol medium was reached at a later time, probably between 72 and 96 hours. The final yield in both instances was approximately 10 per cent. However, the final yields of cellulose in the cultures containing glucose was higher than that usually obtained with this medium.

The fall and rise of the pH characteristic of the cultures containing glucose did not occur in media containing mannitol. In these the pH increased only half of a pH unit, from 6 to 6.5, during the entire 192 hours of incubation. Fructose, known to be produced by A. xylinum through the oxidation of mannitol, apparently was not metabolized to acidic substances.

A slight decrease in the weight of cellulose in older cultures, evident in the cultures containing mannitol, had been previously observed with cultures containing glucose (Table 9). The decrease is slight and may have no significance. However, it is believed to be associated with the loss of vitality of the culture rather than a reversal of the cellulose synthesizing reaction.

The Influence of Adaptation of the Inocula on Cellulose Production by Acetobacter xylinum

If cellulose synthesis by A. xylinum proceeds through a single, low molecular weight precursor, then all of the substrates which can be

TABLE 15

THE RATE OF FORMATION OF CELLULOSE BY A. xylinum IN A MEDIUM CONTAINING GLUCOSE

Incubation Period (Hours)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
12	4.4	1.6	1
	4.4	1.8	
	4.4	1.7	
24	3.8	8.4	4.7
	3.8	8.2	
	3.8	8.8	
48	4.5	18.2	10
	4.7	17.7	
	4.5	17.8	
72	5.4	17.3	9.8
	5.5	16.8	
	5.2	18.7	
96	5.4	18.1	9.2
	5.4	17.1	
	4.9	14.6	
120	5.4	17.8	10
	5.5	16.7	
	5.7	19.5	
144	5.7	20.1	10
	5.4	16.4	
	5.6	17.2	

Medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 6

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants containing glucose.

Incubation: At 30°C.

TABLE 16

THE RATE OF FORMATION OF CELLULOSE BY A. xylinum IN A MEDIUM CONTAINING MANNITOL

Incubation Period (Hours)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
12	6.1	1.3	0.8
	6.1	1.2	
	6.1	1.3	
24	6.3	3.5	1.8
	6.3	3.1	
	6.3	3.1	
36	6.3	7.0	4.1
	6.4		
	6.4	7.8	
48	6.4	10.1	6.4
	6.5	12.1	
	6.5	11.9	
96	6.5	17.3	10.3
	6.6	20.9	
	6.5	16.8	
192	6.5	14.4	9.0
	6.5	16.4	
		17.1	

Medium: Yeast extract . . . . . 1 per cent
Mannitol . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 5.9

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants containing mannitol.

Incubation: At 30°C.

converted to cellulose by this organism must be metabolized, to some extent, to this precursor. Those substrates which form the largest amounts of cellulose might be expected to have a structure more closely resembling that of the precursor. Higher yields have been reported from mannitol and fructose than from glucose.[4,31] Since mannitol is oxidized to fructose by A. xylinum, these higher yields might indicate that fructose is closer to the precursor than glucose. [The higher yields might also be caused by other factors, one possibility being the maintenance of a more favorable pH in the fructose medium.] The results obtained when cells which had been grown on one substrate were inoculated into media containing the other substrates might provide clues to the pathway of cellulose synthesis.

Three media were prepared which were identical except that different carbohydrate substrates were employed in each. All contained yeast extract (1 per cent), KH_2PO_4 (0.3 per cent) and carbohydrate (1 per cent glucose, mannitol or fructose).

The organisms were grown in stock cultures containing the same carbohydrate as the agar slant to which they were transferred for the preparation of inocula.

Three flasks of each media were inoculated with cells which had been adapted to each of the three substrates. Cultures were incubated for 48 hours, and cellulose content and final pH values were determined.

The final pH values were uniform in each medium and, evidently, were little affected by the adaptation of the inocula to different carbohydrates (Table 17). Also, there were only slight variations

TABLE 17

THE INFLUENCE OF THE ADAPTATION OF THE INOCULA ON CELLULOSE FORMATION BY *A. xylinum* IN GLUCOSE, MANNITOL AND FRUCTOSE MEDIA

Test Medium	Cells Adapted to	pH (final)	Cellulose (mgs.)	Yield, % Average
Glucose	Glucose	4.2	14.0	8.1
			14.0	
			15.6	
	Fructose	4.05	14.5	8.0
			14.3	
			14.0	
	Mannitol	4.1	13.6	7.0
			12.1	
			12.0	
Fructose	Glucose	6.7	12.5	7.0
		6.3	15.9	
		7.1	9.5	
	Fructose	6.2	18.7	10.5
		6.2	19.0	
		6.2	19.0	
	Mannitol	6.2	11.3	6.4
		6.2	12.4	
		6.2	10.9	
Mannitol	Glucose	6.3	13.5	7.6
		6.3	13.7	
		6.3	13.5	
	Fructose	6.3	17.8	10.0
		6.3	18.1	
		6.3	18.7	
	Mannitol	6.25	15.3	8.2
		6.25	15.2	
		6.25	14.6	

Basal medium: Yeast extract . . . . . 1 per cent
KH_2PO_4 . . . . . .0.3 per cent
pH 6

Inocula: One milliliter transfers from a suspension of a three-day growth on an agar slant containing the hexose to which the cells were adapted.

Incubation: Forty-eight hours at 30°C.

in cellulose yields from cultures containing glucose. However, definite differences, probably attributable to adaptation of the inocula, were obtained in media containing mannitol or fructose.

Adaptation of the cells to glucose appeared to have little influence on their ability to produce cellulose from the three substrates. Adaptation to fructose improved the cellulose production from fructose and mannitol. Cells which were grown in mannitol, unexpectedly, formed less cellulose in the mannitol medium than did the cells grown in fructose. Also, mannitol-adapted cells formed the least amount of cellulose in the fructose and glucose media. These results indicate that the ability of cells to synthesize cellulose is somehow decreased when they are grown in mannitol. They also provide evidence that the oxidation of mannitol is prerequisite to cellulose formation from this substrate. Otherwise, a greater production of cellulose from mannitol with inocula adapted to fructose than with inocula adapted to mannitol would hardly be expected.

Since cellulose was determined only after 48 hours incubation, it cannot be known whether the amounts of cellulose obtained represented the maximum yields in the cultures. Studies of the rate of cellulose formation and adaptation of the cells to a larger number of substrates would be desirable if experiments of this type were undertaken in the future.

Exploratory Experiments on the Effect of Metabolic Inhibitors on Cellulose Production by *Acetobacter xylinum*

An investigation of the influence of inhibitors on cellulose production by *A. xylinum* was incidental to the major part of this dissertation. Inhibition studies, particularly ones involving competitive inhibition by compounds structurally similar to substrates known to be converted to cellulose, should be informative. However, their value would be enhanced if they were conducted in chemically defined media or with enzyme preparations.

The modes of action of three of the inhibitors employed here have been extensively studied. The cyanide ion is inhibitory to enzyme systems dependent on metallo-proteins such as occur in the cytochrome system.[20] Enzymes, including phosphatases, lipases and carboxylases, requiring metal activators are inhibited by fluoride ion.[28] The competitive inhibition by malonic acid of succinic dehydrogenase in the Krebs cycle of oxidative metabolism has been throughly investigated.[29] The role of bisulfite ion is not so well defined. It is known to inhibit cysteine desulfhydrase[21] and effects due to its ability to add to aldehydic compounds and to its potential for oxidation or reduction might be expected.

The media used in these experiments were similar to the standard medium (p. 9) except that mannitol was substituted for glucose. Stock cultures which were used for inocula varied in age between experiments but were of uniform age for individual experiments. Membranes were treated with 1 per cent NaOH by boiling under reflux for 8 to 10 hours.

Neither initial nor final pH values were determined in these cultures.

No effect on cellulose yield was observed in cultures to which KCN, NaF and malonic acid were added separately at 0.003 M concentrations (Table 18). The inhibitors were added after three days of incubation.

TABLE 18

CELLULOSE PRODUCED IN CULTURES CONTAINING KCN, NaF AND MALONIC ACID ADDED AFTER THREE DAYS INCUBATION

Cellulose in Controls (mg.)		Cellulose in Test Cultures after 7 Days (mg.)		
After 3 Days	After 7 Days	KCN (0.003 M)	NaF (0.003 M)	Malonic Acid (0.003 M)
13.8	26.4	26.8	25.8	27.6
14.3	21.3	25.3	25.6	23.8
		29.6	26.0	21.6

Basal medium: Yeast extract . . . . . 1 per cent
Mannitol . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 6

Inocula: One milliliter transfers from stock cultures

Incubation: At 30°C.

When the same inhibitors were added at the same concentrations to cultures which had been incubated only 24 hours, no cellulose was obtained in the cultures which received KCN (Table 19). Malonic acid and NaF had no effect.

TABLE 19

CELLULOSE PRODUCED IN CULTURES CONTAINING KCN, NaF AND MALONIC ACID ADDED AFTER 24 HOURS INCUBATION

Cellulose in Controls (mg.) After 11 Days	Cellulose in Test Cultures After 11 Days (mg.)		
	KCN (0.003 M)	NaF (0.003 M)	Malonic Acid (0.003 M)
9.9	No	18.5	19.8
18.4		18.1	19.5
17.7	Cellulose	17.5	20.2
20.9	Formed		

Basal medium: Yeast extract . . . . . 1 per cent
Mannitol . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 6

Inocula: One milliliter transfers from stock cultures

Incubation: At 30°C.

Sodium bisulfite (0.05 M and 0.005 M) gave practically complete inhibition of cellulose production when introduced into mannitol-containing cultures after 2 days incubation (Table 20).

An apparent stimulation of cellulose formation was observed with 0.005 M malonic acid (Table 21). The cultures, containing mannitol, had been inoculated 24 hours prior to the addition of malonic acid.

The information obtained in these experiments was too meager for interpretation. This is particularly true since effects on cellulose production could not be differentiated from effects on overall cell metabolism. The stimulation of cellulose formation apparently due to malonic acid was considered to be worthy of further investigation.

TABLE 20

CELLULOSE PRODUCED IN CULTURES CONTAINING $NaHSO_3$ ADDED AFTER TWO DAYS INCUBATION

Cellulose in Controls (mg.)		Cellulose in Test Cultures After 11 Days (mg.)	
After 2 Days	After 11 Days	$NaHSO_3$ (0.05 M)	$NaHSO_3$ (0.005 M)
8.0	21.5	7.3	9.1
11.0	24.2	6.1	6.1
9.2	22.8	7.3	8.4

Basal medium: Yeast extract . . . . . 1 per cent
Mannitol . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 6

Inocula: One milliliter transfers from stock cultures

Incubation: At 30°C.

TABLE 21

CELLULOSE PRODUCED IN CULTURES CONTAINING MALONIC ACID ADDED AFTER 24 HOURS INCUBATION

Cellulose in Controls After 10 Days (mg.)	Cellulose in Test Cultures After 10 Days (mg.) Malonic Acid 0.005 M
16.2	21.5
14.0	24.7
17.5	22.0

Basal medium: Yeast extract . . . . . 1 per cent
Mannitol . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
pH 6

Inocula: One milliliter transfers from stock cultures

Incubation: At 30°C.

The Influence of Malonate on Cellulose Production in Media Containing Glucose, Fructose and Mannitol

A stimulatory effect of malonic acid on cellulose synthesis in media containing mannitol had been noted in the experiments with metabolic inhibitors (Table 21). A broader investigation was undertaken to verify these results and to extend the study to media containing glucose and fructose.

The basal media employed were the same as those used in the adaptation experiments (p. 42). Malonic acid solutions were prepared and adjusted to pH 6 with KOH. Half of the test media received malonate at three levels of concentration (0.0024 M; 0.0048 M; 0.0072 M) before inoculation. The addition was made to the other half of the cultures after 12 hours incubation. At this time volumes of sterile, distilled water equal to the volumes of malonate solution used were added to those flasks not receiving malonate. Thus, all the cultures were handled in a uniform manner.

The inocula were taken from cell suspensions prepared from a three-day growth on agar slants. The agar slants each contained the same carbohydrate as did the medium into which the cells were transferred in order to assure adaptation to the individual substrates.

The cultures were incubated a total of 48 hours. Cellulose weight was determined in each culture and a final pH value obtained on the composite media from triplicate cultures.

The amount of cellulose produced in the cultures containing fructose and mannitol was very small. The average amount in triplicate cul-

tures did not exceed 4.5 mg., and the maximum yield in a single culture was 6.4 mg. The reason for this decreased yield is not known, but it is believed to be associated in some manner with the disturbance of the membranes at the 12-hour addition of malonate and water. The amount of cellulose produced in cultures containing glucose was also less than normal (7.8, 10.3 and 9.7 mg. in the controls), but here the effect was less pronounced.

Though a slight increase in cellulose with increasing malonate concentration appeared in the glucose media, an interpretation of the results of the experiment seemed useless in view of the low yields noted above. The experiment was therefore repeated, omitting the 12-hour addition of malonate to avoid disturbing the cultures during incubation.

The weights of cellulose produced in this experiment were closer to the normal values (Table 22). The cellulose production, however, in the cultures containing fructose were again somewhat low.

Cultures containing mannitol were essentially unaffected by the addition of malonate. A slight downward trend in membrane weights in cultures containing fructose and a slight upward trend in cultures containing glucose corresponded to increasing malonate concentrations in these media.

The final pH values also appeared to be influenced by the presence of malonate. A small decrease in these values with increasing concentration of malonate was evident in cultures containing mannitol and fructose. An opposite effect was noted in the glucose medium.

TABLE 22

THE INFLUENCE OF MALONIC ACID ON CELLULOSE PRODUCTION IN CULTURES OF *A. xylinum*

Malonic Acid Concentration (molar)	pH (final)			Cellulose Produced (mg.)			Cellulose Yield (Avg. %)		
	M[a]	F	G	M	F	G	M	F	G
0	6.3	6.0	4.1	20.6 19.0 19.4	14.0 13.7 13.7	12.6 12.4 23.1	11.1	7.7	6.9
0.0024	6.3	5.9	4.1	20.2 19.3 19.8	11.9 13.1 13.0	13.9 14.2 13.2	11.2	7.1	7.7
0.0048	6.25	5.85	4.3	19.6 18.7 20.0	12.0 15.2 12.1	16.2 15.5 15.5	10.9	6.7	8.7
0.0072	6.2	5.9	4.9	20.1 19.5 18.3	11.5 11.6 11.1	16.4 24.4 16.7	10.8	6.3	9.2

[a] M = Mannitol, F = Fructose, G = Glucose.

Basal Medium: Yeast extract . . . . . 1 per cent
KH_2PO_4 . . . . . 0.3 per cent
Mannitol, fructose or glucose added at 1 per cent concentration. Malonic acid solution, adjusted to pH 6 with KOH, was added before inoculation.

Inocula: One milliliter transfers from cell suspensions prepared from a 3-day growth on agar slants. The carbohydrate in the slants was the same as the media inoculated.

Incubation: Forty-eight hours at 30°C.

The influence of malonate on cellulose production by *A. xylinum* was much smaller than anticipated on the basis of the preliminary experiment. Contrary to expectations, the greatest effect was obtained in glucose media, and this effect could be explained by a buffering action of the malonate similar to that observed with increasing phosphate concentrations (Table 13). Only the effects of malonate present in the media before inoculation can be considered to have been satisfactorily demonstrated. The effects of a post-inoculation addition of malonate remain in doubt.

CHAPTER III

THE PRODUCTION OF CELLULOSE BY *Acetobacter xylinum* IN CHEMICALLY DEFINED MEDIA

Preliminary Observations

Tarr and Hibbert[31] reported cellulose formation by *A. xylinum* in chemically defined media containing L-asparagine as the nitrogen source. Early attempts in this research to reproduce their results were unsuccessful. No immediate explanation of this failure could be made. However, the procedures employed were not completely identical. Tarr and Hibbert used yeast water in their stock cultures and inoculated the asparagine medium with a dilution of a stock culture. On the other hand, in this research yeast extract was employed in the stock cultures, and washed cells from these cultures served as inocula. A reason for the failure to obtain growth might be the exclusion from the medium, or removal from the cells by the washing procedure, of some necessary growth factors.

Experiments were performed to determine the effect of added growth factors on cellulose production in the asparagine medium. The basal medium contained L-asparagine (0.1 per cent), glucose (1 per cent), KH_2PO_4 (0.5 per cent) and NaCl (0.1 per cent). The compounds to be tested were added to approximately 10 ml. volumes of this medium in test tubes. A suspension of washed cells from a stock culture provided

the inocula. Tubes of medium containing yeast extract were inoculated along with the test cultures to insure the ability of the cells to produce cellulose.

Of the compounds tested in this manner, p-aminobenzoic acid, calcium pantothenate and folic acid resulted in growth and pellicle formation (Table 23). Growth, but no pellicle, was obtained in tubes to which a combination of thiamin, riboflavin, niacin and pyridoxine had been added. The addition of purines or pyrimidines did not result in growth.

The choice by Tarr and Hibbert of L-asparagine as a nitrogen source appeared to be quite arbitrary. Other amino acids might serve equally well in this capacity, and the following experiment was intended to test this possibility.

A basal medium consisting of glucose (1 per cent), KH_2PO_4 (0.3 per cent), calcium pantothenate (0.01 per cent) and folic acid (0.008 per cent) was prepared and sterilized by filtration. The following amino acids were added to 10 ml. volumes of this medium at the concentrations indicated:

L-Arginine monohydrochloride . . . 1 mg./ml.
DL-Alanine . . . . . 1 mg./ml.
L-Proline . . . . . 1 mg./ml.
DL-Serine . . . . . 1 mg./ml.
DL-Threonine . . . . . 1 mg./ml.
Glycine . . . . . 1 mg./ml.
DL-Tryptophane . . . . . 0.5 mg./ml.

TABLE 23

THE INFLUENCE OF GROWTH FACTORS ON GROWTH AND PELLICLE FORMATION OF *A. xylinum*

Compound Added	Concentration (mg./ml.)	Response[a]
1. Thiamin nitrate	0.016	
Riboflavin	0.016	
Niacin	0.5	
Pyridoxine	0.01	±
2. p-Aminobenzoic acid	0.1	+
3. Calcium pantothenate	0.1	+
4. Folic acid	0.1	+
5. Rutin	0.03	-
6. Adenine sulfate	0.03	-
7. Cytosine	0.03	-
8. Hypoxanthine	0.03	-
9. Uracil	0.03	-
10. Guanine	0.1	-
11. Xanthine	0.1	-
12. Combination of 3, 6, 7, 8 and 9	0.03 (each)	-
13. Combination of 2, 3, 4, 10 and 11	0.04 (each)	-
14. Combination of 3 and 4	0.08 (each)	+

[a] + Indicates growth and pellicle formation; - indicates neither growth nor pellicle formation; ± indicates growth only.

Basal medium:	L-Asparagine	0.1 per cent
	Glucose	1 per cent
	NaCl	0.1 per cent
	KH_2PO_4	0.5 per cent
Inocula:	One milliliter transfers from a suspension of washed cells prepared from stock cultures	
Incubation	At 30° C. The tubes were observed for periods up to 10 days.	

These tubes, together with control tubes containing only the basal medium, were inoculated with a suspension of washed cells from a stock culture.

Cell increase and pellicle formation occurred in all tubes except that to which DL-threonine had been added. Growth alone was obtained in this tube. Unfortunately, however, growth and pellicle formation was found in the control tube to which no amino acid had been added. An explanation of this result may lie in the utilization of nitrogenous compounds made available by lysis of the cells of the inoculum. The abnormally high concentration of the vitamins might also have been a factor.

Although it appeared that other amino acids might serve as well as asparagine as a nitrogen source for A. xylinum, a complete investigation of this matter was outside the scope of this research.

The Influence of Calcium Pantothenate, p-Aminobenzoic Acid and Niacin on Cellulose Production in Chemically Defined Media

Rao and Stokes[26] reported on the nutritional requirements of several species of the genus Acetobacter. They found that A. xylinum produced a pellicle when grown in a medium containing only ammonium sulfate, glucose, phosphate and minerals. The inocula were taken from cell suspensions prepared from the growth on agar slants containing yeast autolysate. They also reported that strains of A. suboxydans exhibited demands for growth factors which could be satisfied by pantothenic, p-aminobenzoic and nicotinic acids.

An investigation of the effects of these vitamins on cellulose production by A. xylinum was undertaken. The same quantitative technique was employed which had been used in experiments with media containing yeast extract.

The basal medium for the first of these experiments differed in several respects from that used in the preliminary work (p. 52). The concentration of glucose was increased to 2 per cent, and ethanol (0.5 per cent) and trace elements were included in the medium. The trace element solution had the following composition: $MgSO_4 \cdot 7\ H_2O$, 10 g.; $MnSO_4 \cdot H_2O$, 0.1 g.; $FeSO_4 \cdot 7\ H_2O$, 0.1 g.; and HCl (conc.), 3 ml., made to 100 ml. with distilled water. One milliliter of this solution was added to 1 liter of medium. The vitamins were introduced into the culture flasks before inoculation. Their concentrations, separately and in combination, in the media were for calcium pantothenate and p-aminobenzoic acids, 1 gamma per ml., and for niacin, 2 gammas per ml.

The initial pH was 6 in every case except that a duplicate of cultures containing all the vitamins was adjusted to pH 6.8. The inocula were 1 ml. transfers from a suspension of a 3-day growth on agar slants. The cultures were incubated 48 hours before final pH values and cellulose weights were determined.

Cellulose, in contradiction of the results of preliminary experiments, formed in cultures which had received no vitamins (Table 24). The amount of cellulose produced in media which contained a combination of calcium pantothenate and niacin or of the three vitamins was slightly greater than in the other cultures. A medium containing niacin alone was not tested.

TABLE 24

THE INFLUENCE OF CALCIUM PANTOTHENATE, p-AMINOBENZOIC ACID AND NIACIN ON CELLULOSE PRODUCTION (UNWASHED INOCULA)

Calcium Pantothenate gamma/ml.	p-Aminobenzoic Acid gamma/ml.	Niacin gamma/ml.	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
0	0	0	2.8 2.8	6.4 6.8	3.7
1	0	2	2.8 2.8	7.4 8.2	4.3
0	1	2	2.8 2.8	6.4 6.8	3.7
1	1	2	2.8 2.8	8.4 8.1	4.6
1[a]	1	2	3.2 2.9	8.6 8.7	4.8

[a] 0.03 M phosphate, pH 6.8.

Basal medium:
- L-Asparagine . . . . . 0.1 per cent
- Glucose . . . . . 2.0 per cent
- Ethanol . . . . . 0.5 per cent
- Phosphate . . . . . 0.022 M
- pH 6
- Trace element solution [$MgSO_4 \cdot 7H_2O$, 10 g.; $MnSO_4 \cdot H_2O$, 0.1 g.; $FeSO_4 \cdot 7H_2O$; 0.1 g.; HCl (conc.), 3 ml., made to 100 ml. with distilled H_2O.] . . 1 ml./liter

Inocula: One milliliter of cell suspension from a 3-day growth on an agar slant

Incubation: Forty-eight hours at 30°C.

The higher initial pH in the cultures containing all three vitamins seemed to have little influence on cellulose production.

An explanation was needed for the production of cellulose in the basal medium where previous attempts had failed. The changes, noted above, which had been made in the medium were not considered to be sufficient to induce cellulose formation. The use of unwashed cells in the inocula seemed to be a more likely cause. Since there was some evidence that calcium pantothenate or niacin had a stimulatory effect on cellulose formation, the removal of growth factors during the washing of the inocula was a plausible explanation for the previous failures. It was decided, therefore, to determine what effect the washing of the cells before inoculation would have on cellulose production.

The basal medium used in the previous experiment was inoculated with cells which had received various degrees of washing. Thus, cells suspended in water directly from the agar slants were used first. The cells were then removed from suspension by centrifugation and resuspended in 8 ml. of water. Two 1 ml. portions of this suspension were used as inocula. This process was repeated a second and third time, reducing the volume of the water added after each centrifugation. The cultures were incubated 72 hours to allow for differences in the rate of cellulose formation due to possible differences in the size of inocula.

The amount of cellulose produced in cultures inoculated from the original suspension was 7.3 and 5.3 mg. (Table 25). Media inoculated with cells which had received one washing produced only 1.8 and 1.9 mg. of cellulose. A further decrease was obtained with more

TABLE 25

CELLULOSE PRODUCTION WITH WASHED CELLS AS INOCULA

Treatment of Inocula	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
Cells not washed	2.8 2.9	7.3 5.3	3.5
Cells washed once	3.3 3.2	1.9 1.8	1.0
Cells washed twice	3.8 3.2	1.8 1.2	0.83
Cells washed three times	3.4 3.8	0.3 -	0.2

Basal medium: L-Asparagine . . . . . 0.1 per cent
Glucose . . . . . 2.0 per cent
Ethanol . . . . . 0.5 per cent
Phosphate . . . . . 0.022 M
Trace element solution (p. 57).. 1 ml./liter
pH 6.1

Inocula: One milliliter of cell suspension from a 3-day growth on agar slants

Incubation: Seventy-two hours at 30°C.

completely washed cells, but some cellulose was formed in all the cultures.

These results indicated the advisability of using washed inocula in determining the growth factor requirement of microörganisms.

The experiment with calcium pantothenate, p-aminobenzoic acid and niacin was repeated using inocula which had received the complete washing treatment described previously. The concentration of glucose in the basal medium was reduced to 1 per cent and ethanol was omitted. These changes were made to reduce the difference between this medium and that used in preliminary experiments. Cultures containing only niacin were included. The concentrations of the vitamins were the same as previously employed. The cultures were incubated 48 hours before determinations of final pH values and cellulose weight were made.

The difference in the amount of cellulose produced in cultures containing calcium pantothenate and in the controls was much more pronounced in this experiment (Table 26). The controls contained 3.1 and 2.8 mg. of cellulose, while 7.5 and 6.7 mg. were present in the cultures to which calcium pantothenate had been added. p-Aminobenzoic acid gave a smaller but definite increase (5.1 and 4.6 mg.), niacin showed a doubtful stimulation (2.5 and 4.5 mg.), and a combination of the three vitamins appeared to be no better than calcium pantothenate alone.

The growth factors which were evidently present in the original cell suspension may have had their origin in the A. xylinum cells, or they may have come from the yeast extract in the slant. If they did

TABLE 26

THE INFLUENCE OF CALCIUM PANTOTHENATE, p-AMINOBENZOIC ACID AND NIACIN ON CELLULOSE PRODUCTION (WASHED INOCULA)

Calcium Pantothenate gamma/ml.	p-Aminobenzoic Acid gamma/ml.	Niacin gamma/ml.	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg. %)
0	0	0	3.3 3.3	3.1 2.8	1.6
1	0	0	3.1 3.1	7.5 6.7	4.0
0	1	0	3.1 3.1	5.1 4.6	2.7
0	0	2	3.3 3.1	2.5 4.5	1.9
1	1	2	3.1 3.1	6.9 7.0	3.9

Basal medium:

L-Asparagine . . . . . 0.1 per cent

Glucose . . . . . 1.0 per cent

Phosphate . . . . . 0.022 M

Trace Element Solution (p.57)...1 ml./liter

pH 6

Inocula: One milliliter of cell suspension from a 3-day growth on agar slants; cells washed by centrifugation in four portions of sterile, distilled water.

Incubation: Forty-eight hours at 30°C.

originate in the cells, then they might also be present in the liquid media in which cellulose had been formed by A. xylinum. This "exhausted medium" would be expected to increase cellulose production when combined with fresh medium and inoculated with washed cells.

An unwashed cell suspension from an agar slant was used to inoculate 6 flasks of asparagine medium. The medium contained L-asparagine (0.1 per cent), phosphate (0.018 M KH_2PO_4; 0.004 M K_2HPO; pH 6.5) and trace elements. After incubation for 48 hours membranes were removed from the cultures, processed and weighed. The weights obtained were 5.5, 5.5, 10.2, 6.4, 5.3, and 6.5 mg.

The media (pH 3.1) remaining in the flasks were combined and filtered through an ultra-fine sintered glass filter after pH had been adjusted to 6.5. The filtrate and the original medium (10 ml. of each) were combined and inoculated with washed cells. Flasks of the original medium, as controls, were also inoculated with washed cells. The cultures were incubated 48 hours.

The final pH of the control culture was 3.5, and the combined weight of two membranes was 3.9 mg. The final pH of the test cultures was 4.6, and two membranes had a combined weight of 1.2 mg.

If substances stimulatory to cellulose formation are contained in "exhausted" A. xylinum cultures, their presence was not revealed by the above experiment. The addition of fresh medium to the residue obtained from lyophilization of the culture filtrate should provide a better method for testing for these substances.

Calcium pantothenate and p-aminobenzoic acid appear to be definitely stimulatory to the growth and/or cellulose production by A. xylinum. However, the effect of varying the concentrations of these vitamins was not determined. This, and the possible effects of folic acid, niacin and other growth factors, should probably be investigated using much smaller inocula than were employed here.

The Influence of Glucose Concentration on Cellulose Production in Chemically Defined Media

The effect of glucose concentration on the yield of cellulose in media containing yeast extract was made somewhat uncertain by the formation of cellulose from yeast extract alone (Table 11). On the other hand, cellulose produced in chemically defined media should have its origin entirely in the glucose. The latter situation would be most desirable for experiments involving specifically labeled carbohydrate substrates.

A basal medium (pH 6) containing L-asparagine (0.1 per cent), phosphate (0.022 M) and trace elements was used for determining the effect of glucose concentration on cellulose formation. Glucose was added at four concentrations (0.05, 0.1, 0.2 and 1 per cent) to four sets of duplicate flasks of this medium. Another series was prepared, identical with this except that ethanol (0.5 per cent) was added to each flask. The media were inoculated with cell suspension (unwashed), and the cultures were incubated for 48 hours before the final pH and cellulose weights were determined.

The amount of cellulose in cultures not containing ethanol varied from 2 mg. at 0.05 per cent glucose to 4 mg. at 1 per cent glucose. The former represented a cellulose yield of 23 per cent of the theoretical. The inclusion of ethanol in the medium had little or no effect on cellulose production in cultures containing 0.05 per cent glucose. However, the yields of glucose in cultures with 0.1, 0.2 and 1 per cent glucose were approximately doubled by the addition of ethanol. The highest percentage of the theoretical yield (26 per cent) was obtained in cultures containing ethanol and 0.1 per cent glucose. (Table 27).

The amount of asparagine (0.1 per cent) employed in these media is believed to provide insufficient nitrogen for the growth of A. xylinum. The above yields would probably be improved by an increase in available nitrogen from asparagine or from other, perhaps more efficient, sources.

TABLE 27

THE INFLUENCE OF GLUCOSE CONCENTRATION ON CELLULOSE PRODUCTION by *A. xylinum* IN CHEMICALLY DEFINED MEDIA

Glucose Concentration (%)	Ethanol (%)	pH (final)	Cellulose Produced (mg.)	Cellulose Yield (Avg.%)
0.05	0	5.8 5.8	2.2 2.0	23
0.10	0	4.2 4.5	- 2.5	14
0.20	0	3.8 3.7	3.1 2.1	7
1.0	0	3.1 3.1	3.6 4.3	2
0.05	0.5	4.1 4.0	1.6 2.8	24
0.10	0.5	3.7 3.7	4.6 4.7	26
0.20	0.5	3.6 3.6	6.2 5.3	15
1.0	0.5	3.0 3.0	6.9 6.5	4

Basal medium: L-Asparagine . . . . . 0.1 per cent
Phosphate . . . . . 0.022 M
Trace Element Solution (p. 57) . . 1 ml./liter
pH 6

Inocula: One milliliter transfers from a suspension of unwashed cells from a 3-day growth on agar slants

Incubation: Forty-eight hours at 30°C.

CHAPTER IV

DISCUSSION

The Influence of pH on Cellulose Production

The effect of the initial pH of the medium on cellulose formation in cultures containing glucose is very pronounced. Thus, one-unit increases of the initial pH between pH 4 and pH 7 resulted in 90, 26 and 50 per cent increases in the yields of cellulose. The initial pH which is optimum for cellulose production in a medium containing glucose (1 per cent), yeast extract (1 per cent) and phosphate buffer (0.022 M) is approximately 7. It is believed, however, that this statement of the optimum should be further amplified to include the size of the inoculum employed.

The following reasoning lies behind this belief. The failure in this research to propagate A. xylinum in stock culture at pH 7 and the report of Powell[25] that no growth occurred in media at this pH are in contradiction to the above statement that pH 7 is an optimum for cellulose production. A logical explanation of this discrepancy may lie in the size of the inoculum, since this was the only obvious difference between the experimental conditions employed. It is postulated, therefore, that the large inoculum used in the successful experiment was capable of rapidly reducing the pH of the medium to a value at which the organism could grow. The relatively small inoculum from a stock culture might not have been able to reduce the pH sufficiently to permit

growth. If this is true, the rate and possibly the magnitude of the pH reduction depends on the size of the inoculum and, consequently, the value of the initial pH for maximum cellulose production also depends on the inoculum size.

The actual pH optimum for the cellulose synthesizing reaction could not be determined because of the extreme variation of the pH during the incubation of cultures containing glucose. It is estimated to be located between pH 5 and pH 7, judging by the initial and final pH values of the cultures producing the larger amounts of cellulose. A more exact value could probably be determined with media containing mannitol or fructose, in which there are much smaller changes in the pH.

An increase in cellulose yield produced by an increase of the initial pH might result from a greater number of cells being produced or from a larger amount of cellulose being formed per cell. The latter situation would indicate an influence of pH over the rate of the cellulose synthesizing reaction. In the absence of a method for the simultaneous measurement of cell increase and cellulose weight, the existence of the latter phenomenon can only be inferred. Thus, the following observations may indicate that a pH of 7 in glucose media increases the rate of the synthetic reaction rather than increasing the population.

The failure to obtain growth in stock cultures at pH 7 and the report of Powell which supports this observation have been previously mentioned. These results, though in need of additional experimental confirmation, provide some evidence that pH 7 is inhibitory to the

growth of A. xylinum. If this hypothesis of inhibition is accepted, then it might further be expected that the adjustment of a growing culture to pH 7 would not result in a rapid increase of population. A corollary to this deduction is that if cellulose formation is increased by the above adjustment of pH, it should result from an increase in the rate of reaction for cellulose synthesis.

Assuming the above reasoning is correct, the increased cellulose yield that was obtained through the post-inoculation adjustment of the pH represents a greater production of cellulose per cell. This deduction, hypothetical though it may be, appears to have sufficient basis in fact to merit further investigation.

The Influence of the Concentration of Media Components on Cellulose Production

The changes in cellulose production occurring when the concentrations of media components are varied appear to be due largely to effects on the pH of the media. Evidence for this is provided by the correlation between the cellulose yields and the final pH values. Thus, in cultures containing more than 0.5 per cent glucose, a decrease in the final pH occurs concurrently with a decrease in the amounts of cellulose produced. Simultaneous increases in cellulose yields and final pH values are obtained by increasing the yeast extract concentrations from 0.5 to 2.5 per cent. A similar correlation is observed with increasing phosphate concentrations.

There are, obviously, factors other than pH effects concerned in the influence of glucose, yeast extract and phosphate concentration

on cellulose production. This is apparent in the data for glucose and yeast extract. The influence of phosphate concentration, however, is almost entirely explainable by an increased buffering action. The yeast extract present in these media apparently supplies enough phosphate to satisfy the demands of A. xylinum.

Approximately 0.2 per cent glucose appears to be the concentration which is optimum for cellulose production. Under the experimental conditions employed the highest percentage of the theoretical yield was obtained with this concentration. The best concentration of yeast extract is more difficult to ascertain. The effect of yeast extract on cellulose production at concentrations below approximately 0.5 per cent is probably due to a stimulation of growth. At higher concentrations the pH effect is operative. The glucose concentration greatly influences the pH of the culture and in a direction opposite to that of yeast extract. For this reason, at least at the higher concentrations, these components of the medium cannot be considered as independent variables. Therefore, it is only possible to state that in a medium containing 1 per cent glucose, 2.5 per cent of yeast extract approximates the optimum for the concentration of this component.

Again, the comparative stability of the pH in media containing mannitol or fructose would be a distinct advantage in establishing optima for the concentrations of media components.

The influence of ethanol on cellulose production has been reported previously and is believed to be due to the preferential use of ethanol as an energy source.[31,22] It has also been reported that ethanol

itself is not converted to cellulose by A. xylinum.[30, 10]

Minor, et al.,[22] in their experiments with glucose-1-C^{14}, reported cellulose yields of 4 to 8 per cent from media containing 1 per cent each of glucose and yeast extract and 0.5 per cent ethanol. No attempt was made in this research to determine the effect of adding ethanol to a medium containing glucose and yeast extract at these concentrations. However, in a medium containing 0.2 per cent glucose and 2.5 per cent yeast extract (concentrations indicated by previous experiments to be the optima for these components) the highest yield of cellulose was obtained with 1 per cent ethanol. This yield of 43.6 per cent represents a considerable increase over the yields reported by Minor, et al.

The Rate of Cellulose Formation

The rate of cellulose formation is dependent upon the size of the inoculum. This is to be expected from the greatly increased number of cells that are formed with each generation as the size of the inoculum is increased. However, the total number of cells produced in a culture is controlled by cultural conditions and is the same in identical media even though the size of the inoculum is varied. This would also be true of the amount of cellulose produced if cell increase were the only factor affecting cellulose production.

Total cellulose production was obtained in 48 hours in glucose media with the inocula employed in these experiments. Practically all of the cellulose was formed between the twelfth and thirty-sixth hours

of incubation. This was probably also the period of maximum cell increase, but this could not be demonstrated.

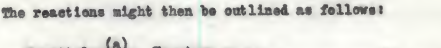
Cellulose formation proceeds at a somewhat slower rate in media containing mannitol than in media containing glucose. Two possible explanations for this are a slower rate of growth and less availability of substrate. The pH of cultures containing mannitol increases one-half of a pH unit from the initial pH of 6 during 48 hours of incubation. This pH might be inhibitory and thus cause a decrease in the growth rate. The hypothesis of a less available substrate is based on the assumption that mannitol must be oxidized to fructose before cellulose formation can take place. A slow rate for this reaction might be reflected in the rate of cellulose formation. These explanations are entirely speculative.

The Influence of Adaptation of the Inocula on Cellulose Production

The knowledge of the mechanism of cellulose formation by A. xylinum is too slight to permit a meaningful interpretation to be made of the adaptation experiment performed in this research. The rather scanty data provided by the single experiment might have some value as a basis for speculation.

Two aspects of the metabolism of the three substrates, glucose, fructose and mannitol, have been fairly well established. These are that oxidation of glucose to gluconic acid and of mannitol to fructose are main energy-yielding reactions in the metabolism of these compounds by A. xylinum.[4] The fate, however, of fructose is obscure.

An examination of the data from the adaptation experiment will be made on the basis of two assumptions concerning the synthesis of cellulose. One is that cellulose synthesis from all substrates proceeds by way of a single, common precursor. The other is that mannitol must be oxidized to fructose before cellulose can be formed from this substrate. The reactions might then be outlined as follows:



On the basis of the first assumption, it might be expected that inocula adapted to glucose would produce less cellulose from fructose than would inocula adapted to fructose, since the enzymes required for reactions (b) and (c) might well be different. This was found to be the case. However, the reverse should also be true; that is, inocula adapted to fructose should produce less cellulose from glucose than inocula adapted to glucose. The yields from glucose were approximately the same from the two inocula.

These results, although providing only very slight confirmation of the first assumption, at least are believed not to be contradictory.

Inocula adapted to fructose produced approximately the same amount of cellulose from mannitol that they did from fructose. This might be considered to be some confirmation for the second assumption, since cells grown on fructose should be adapted to reaction (b) whether or not it is preceded by reaction (a). However, cells adapted to mannitol produced less cellulose from mannitol than did cells adapted to fructose.

This was rather unexpected, since on the basis of the second assumption, fructose is also present in mannitol media to permit adaptation to reaction (b). An explanation might lie in the growth of the cells on an agar slant prior to inoculation. This aerobic condition of growth might have prevented the accumulation of fructose, and an exogenous supply of fructose might be necessary for adaptation.

This single experiment provides little information even for speculation. However, more carefully planned experiments extended to include other substrates and involving studies of the rate of cellulose formation might prove valuable.

The Influence of Inhibitors on Cellulose Formation

The cyanide ion is an inhibitor for the cytochrome enzymes which have an iron porphyrin as a prosthetic group. This provides a probable explanation of the inhibition of the production of cellulose when KCN is added after 24 hours incubation to cultures of A. xylinum. The failure to obtain inhibition when cyanide is added after three days incubation could possibly be due to an insufficient concentration of the inhibitor for the greatly increased number of cells present at this time.

The lack of inhibition by the fluoride ion might indicate that enolase or other enzymes requiring metal activators are not involved in the metabolism of A. xylinum. (Mannitol was the only substrate used in these experiments with inorganic inhibitors.)

Many reasons might be advanced for the inhibition produced by the bisulfite ion, but no basis for a choice among them is provided by the single experiment performed.

The effect of malonic acid when added after inoculation needs further clarification. The slight stimulation by malonate of cellulose production in cultures containing glucose is another of the effects that can probably be attributed to an influence on the pH of the culture.

The Effect of Calcium Pantothenate, Niacin and p-Aminobenzoic Acid on Cellulose Production

Evidence that folic, pantothenic and p-aminobenzoic acids stimulate the growth of A. xylinum was obtained in the preliminary experiments with growth factors. The report of Rao and Stokes[26] that A. xylinum produces growth and pellicle in the absence of added vitamins was published after these experiments were performed.

It seemed more than coincidental that they found requirements by other species of the genus Acetobacter for nicotinic, p-aminobenzoic and pantothenic acids which included two of the vitamins implicated by the preliminary experiments mentioned above. The explanation of the apparently contradictory results may be the use of unwashed cells for inocula by Rao and Stokes.

Definite stimulation of cellulose production is obtained by the addition of pantothenic and p-aminobenzoic acids to chemically defined media inoculated with washed cells. A similar effect is not produced

by nicotinic acid. However, in a large inoculum, even though washed, there may still be present a sufficient concentration of some of the vitamins to satisfy the requirements of the organism.

An additive stimulatory effect is not produced in media containing both pantothenic and p-aminobenzoic acids. This might be the result of limitations placed on growth by other cultural conditions such as limited nitrogen supply, unfavorable pH, or the absence of other necessary growth factors.

The Influence of Glucose Concentration and Ethanol on Cellulose Production in Chemically Defined Media

Yields of cellulose as high as 26 per cent are obtained in asparagine media with 0.1 per cent glucose and 0.5 per cent ethanol present. This indicates the feasibility of performing experiments with radioactive substrates in a chemically defined medium. The yield of cellulose could probably be increased by the addition of growth factors (unwashed inocula were used) and proper adjustment of the nitrogen source.

Suggestions Concerning Future Research on the Production of Cellulose by A. xylinum

The numerous possibilities for future research on the production of cellulose by A. xylinum will not be considered fully here. However, the use of chemically defined media and a method for relating cell increase to cellulose synthesis are considered to be almost essential to the success of such research.

Clues to the mechanism of cellulose formation might be obtained from a re-examination of the substrates reported to yield cellulose and the establishment, definitely, of their ability or inability to do so. Chromatographic analysis of lyophilized cultures, studies of competitive inhibition by substrate analogs and enzyme adaptation experiments should also provide valuable information.

SUMMARY

The effects of variations of the pH and of the concentrations of media components on cellulose formation by A. xylinum were determined in media containing glucose. The initial pH of the medium was found to have a profound effect on cellulose production. Also, a post-inoculation adjustment of the pH of a culture to pH 7 resulted in a marked increase in the yield of cellulose. The influence of the concentrations of glucose, yeast extract and phosphate could, in a large measure, be attributed to effects on the pH of the culture. The increase in yield produced by the addition of ethanol to the media was apparently not due to the pH effect.

The highest yield of cellulose, 43.6 per cent, was obtained in a medium containing 0.2 per cent glucose, 2.5 per cent yeast extract and 1 per cent ethanol. This medium was adjusted to pH 6.7 with 0.044 M phosphate.

Total cellulose production in cultures containing glucose was reached within 48 hours. The rate of cellulose formation in media containing mannitol was slower, total yield being obtained after 72 to 96 hours.

Inocula adapted to fructose produced the highest yields of cellulose from mannitol and fructose. This was believed to indicate that the oxidation of mannitol to fructose was necessary before cellulose could

be formed in this medium. The results of the experiments are believed to point out the value of employing the techniques of simultaneous adaptation in future research.

Under the conditions of the experiments, cyanide and bisulfite ions were inhibitory to cellulose production in media containing mannitol, while fluoride ion was without effect. Malonic acid had little influence on cellulose production when present in the media before inoculation. A slight increase in cellulose yields obtained with malonic acid in a glucose medium was attributed to the pH effect.

Pantothenic and p-aminobensoic acids were found to be definitely stimulatory to cellulose production in a chemically defined medium.

A cellulose yield of 26 per cent obtained in a chemically defined medium indicated that experiments with radioactive labeled substrates would be feasible in such a medium.

APPENDIX I

DIFFICULTIES ENCOUNTERED IN ENDEAVORING TO DETERMINE CELL GROWTH IN CULTURES OF *Acetobacter xylinum*

The liquid below the membrane in an undisturbed culture of *A. xylinum* appears clear and non-turbid. Practically all of the cells in the culture are imbedded in or attached to the cellulose membrane. This situation makes the simultaneous determination of cellulose production and cell growth extremely difficult, if not impossible.

When a culture was shaken vigorously cells were loosened from the membrane and a marked turbidity developed. This turbidity was measured in many of these experiments in the hope that a correlation with actual cell number could be demonstrated. The procedure for these determinations involved striking the flasks against the hand a uniform number of times and with a uniform stroke. The membranes were then removed. (Absorbed culture liquid was pressed out against the necks of the flasks.) The liquid remaining in the flask was adjusted to the original volume of the culture with distilled water, and the transmission of a 10-ml. sample was determined with an Evelyn Photoelectric Colorimeter. The galvanometer was set at 100 per cent transmission with 10 ml. of the original medium, using the 515 millimicron filter. Galvanometer readings were converted to photometric density by the formula $L = 2 - \log G$, in which L is the symbol for photometric density.

Turbidity determinations on triplicate cultures after increasing periods of incubation gave poor precision (Table 28). Neither was there consistent correlation between these values and the weight of the cellulose produced. Plotting photometric density against incubation time resulted in a curve with little resemblance to a normal growth curve.

The turbidity after 24 hours incubation was slightly lower than after 12 hours incubation. The yield of cellulose, however, was much greater in the 24-hour cultures than in the 12-hour cultures. This indicated that new cells were held fast in the membrane.

This same lack of correlation between turbidity and cellulose yield was found in other experiments where turbidity was measured. When occasionally the values appeared to reflect a logical relation between cell growth and cellulose production, no confidence could be placed in the results.

This lack of confidence was increased by the results of nitrogen determinations. Membranes washed for 24 hours in running water were found to contain 2.3 mg. of nitrogen, while the washed cells from the same culture contained only 0.07 mg. of nitrogen. All of the nitrogen in the membrane may not have represented cellular material, but the ratio of cells in the membrane to cells free in the medium must certainly have been large.

TABLE 28

AN ATTEMPT TO RELATE CELL GROWTH AND CELLULOSE PRODUCTION IN CULTURES OF *A. xylinum*

Incubation Period (hours)	Galvanometer Reading (G)	Photometric Density (L)[a]	Cellulose Produced (mg.)
12	85	0.071	1.6
	87	.061	1.8
	85	.071	1.7
24	86	.066	8.4
	88	.056	8.2
	89	.051	8.8
48	80	.097	18.2
	71	.149	17.7
	84	.076	17.8
72	70	.155	17.3
	76	.129	16.8
	67	.174	18.7
96	70	.155	18.1
	65	.189	17.1
	67	.174	14.6
120	61	.215	17.8
	58	.229	16.7
	60	.222	19.5
144	57	.244	20.1
	60	.222	16.4
	57	.244	17.2

[a] L = 2 − Log G. Turbidity of freshly inoculated media: L = 0.042.

Basal medium: Yeast extract . . . . . 1 per cent
Glucose . . . . . .1 per cent
KH_2PO_4 . . . . . .0.1 per cent
pH 6

Inocula: One milliliter transfers from a suspension of a 3-day growth on agar slants

Incubation: At 30°C.

APPENDIX II

CHEMICALS AND APPARATUS

Nutritional Biochemicals Corporation:

- Cytosine
- Uracil
- Adenine sulfate
- Hypoxanthine
- Guanine hydrochloride
- Xanthine
- Folic acid
- Rutin
- L-Arginine monohydrochloride
- L-Proline
- L-Asparagine
- DL-Tryptophane
- DL-Serine
- DL-Alanine
- D-Levulose

Merck & Co., Inc.:

- Calcium pantothenate
- Riboflavin
- p-Aminobenzoic acid
- DL-Threonine
- Glycine
- Dextrose

Eastman Kodak Company:

- Mannitol
- Malonic acid

Difco Laboratories, Inc.:

- Yeast extract

Dr. W. M. Lauter, College of Pharmacy, University of Florida:

- Niacin
- Pyridoxine
- Thiamin nitrate

Thelco Incubator, Cat. No. 1483, Precision Scientific Co., Chicago, Ill.

Evelyn Photoelectric Colorimeter, Rubicon Co., Philadelphia, Pa.

Beckman Glass Electrode pH Meter, Model H 2, National Technical Laboratories, South Pasadena, California

Seitz Filter, Cat. No. 9-738, Fisher Scientific Co., Pittsburg, Pa.

Erlenmeyer Flasks, 250 ml., Cat. No. 4980, Corning Glass Company, Corning, N. Y.

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BIOGRAPHICAL ITEMS

Howard H. Woeber was born in Wheeling, W. Va., on March 31, 1914. He received his undergraduate education at Kanawha Junior College in Charleston, W. Va., and West Virginia University, Morgantown, W. Va. The Bachelor of Arts and Master of Science degrees with majors in chemistry were received from the latter university.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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